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Are Known Pyrogenic Cytokines Responsible for Fever in Influenza?

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The levels of interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, and macrophage inflammatory protein (MIP)-1α released from human peripheral blood leucocytes (PBL) following interaction with influenza virus clone 7a (virulent, produces high fever in ferrets) and A/Fiji (attenuated, produces relatively low fever in ferrets) were low and similar for the two viruses. Neither strain induced interferon (IFN)-γ, and release of IL-8 (which occurs on incubation of PBLs alone) was reduced after interaction with the two viruses. The levels of IL-1 and IL-6 detected in the plasma of infected ferrets were low and did not correlate with the onset, duration or magnitude of the fevers produced by clone 7a and A/Fiji. Relatively large amounts (100,000 pg/kg) of IL-1 and TNF-α were needed to produce appreciable fever in rabbits, and such quantities of IL-6 were not pyrogenic. Hence, as for previous observations, no evidence could be obtained that induction of known pyrogenic cytokines is responsible for the febrile response in influenza. The possibility that some other mediator(s) may be involved cannot be ruled out. ***J. Med. Virol. 52: 336–340, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

In humans, fever and other constitutional effects of influenza, such as headache, myalgia, and nausea result from the action of endogenous pyrogen (EP) produced by phagocytes in response to virus infection. These constitutional effects vary in severity for different virus strains; more recent H1N1 isolates producing milder illness than H3N2 strains [Coates et al., 1986; Jakeman et al., 1991; Wright et al., 1980]. In ferrets also, two H1N1 strains (A/USSR/90/77 [A/USSR] and A/Fiji/15899/83 [A/Fiji]) produced lower fevers than two virulent H3N2 clones 7a and 64c of the A/England/939/69 × A/PR/8/34 reassortant system [Coates et al., 1986]. In these intranasally infected ferrets, fever resulted from liberation of EP following virus-phagocyte interaction in the upper respiratory tract (URT) [Sweet et al., 1979]. Clones 7a and 64c produced more fever in ferrets than A/USSR or A/Fiji when large quantities of virus were inoculated intravenously [Coates et al., 1986], and they released more EP from nasal inflammatory phagocytes than the H1N1 viruses [Tinsley et al., 1987].

Several known cytokines induce fever in experimental animals, and one or more of them may be the EP produced in influenza virus infection. These cytokines include interleukin (IL)-1 [Dinarello, 1992], IL-6 [Akira & Kishimoto, 1992; Dinarello et al., 1991], tumour necrosis factor (TNF)-α [Vassalli, 1992], interferon (IFN)-γ [Dinarello et al., 1984], IL-8 [Zampronio et al., 1994], and macrophage inflammatory protein (MIP)-1α [Davatelis et al., 1989]. The differences in fever produced by different strains may reflect differential induction of the same or different cytokines.

In previous work [Jakeman et al., 1991] we investigated whether there was any correlation between the levels of IL-1, IL-6, and TNF-α found in the culture supernatants of phagocytes that had interacted with clone 7a and A/Fiji and the degrees of fever produced by intracardiac inoculation of these supernatants into ferrets (i.e., EP content). Two systems were used: incubation of ferret nasal phagocytes in vitro which had been infected in vivo by the two viruses (i.e., nasal washings of infected ferrets), and incubation of human peripheral blood leucocytes (PBL) after infection in vitro with each of the two viruses. In both cases, clone 7a produced more EP than A/Fiji, but there were no significant differences in the amounts of cytokines induced by the two viruses, or any correlation between the amount of one cytokine and the level of EP in the same sample.

We repeated the previous study of cytokines induced

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in human PBLs, and extended it to IL-8, IFN-γ, and MIP-1α. Additionally we examined the levels of IL-1 and IL-6 in the bloodstream of infected ferrets at different stages of the fevers produced by clone 7a and A/Fiji. Finally, to see if the levels of cytokines induced in vitro were capable of producing fever, known doses of recombinant human cytokines were administered to rabbits.

MATERIALS AND METHODS

Viruses

The virulent clone 7a of the A/PR/8/34 × A/England/939/69 (H3N2) reassortant system [Beare & Hall, 1971] and the less virulent A/Fiji/15899/83 (H1N1) strain [Coates et al., 1986] were grown in 10 day embryonated hen's eggs (J.K. Needle & Co., Herts., UK) at 35°C. Allantoic fluid was harvested 48 hr after inoculation and clarified by centrifugation (1,000*g,* 30 min, 4°C). Virus was pelleted by ultracentrifugation (80,000*g,* 90 min, 4°C), mixed with TSE buffer (0.01 M Tris-HCl, 1 mM EDTA, 0.85% NaCl) and left at 4°C overnight. Sucrose solution (57% w/v) was added to the virus suspension to give a final concentration of 38% (w/v) sucrose. It was then layered onto fresh 38% sucrose and centrifuged (80,000*g,* 60 min, 4°C). The virus-containing supernatant was diluted to 31% (w/v) sucrose and centrifuged (80,000*g,* 12–16 hr, 4°C). The virus pellet was resuspended in Dulbecco A PBS and stored at −70°C. Infectivity [50% egg infectious dose (EID_{50})] was determined in 10 day eggs and titres quantified using the method of moving averages.

Separation of Human PBLs and Induction of Cytokines by Virus Infection

Buffy coat preparations obtained from the West Midlands Regional Blood Transfusion Service were diluted 1:2 in RPMI 1640 medium (Gibco, Paisley, UK) and layered onto cushions of Histopaque 1119 (Sigma, Poole, UK). After centrifugation (1,000*g,* 30 min, 37°C), the leucocytes were removed from the interface and washed three times in RPMI 1640. Cell viability was determined by trypan blue exclusion at the same time as the cells were counted. They were resuspended to give 2×10^7 cells/ml. One ml of the PBL suspension was mixed with 1 ml of virus suspension diluted in RPMI 1640 (to give 100 EID_{50}/cell) or with 1 ml of RPMI 1640 alone (for controls). After incubation at 37°C for 60 min, the cells were pelleted, washed three times in RPMI 1640, and resuspended at 2×10^6 cells/ml in RPMI 1640 containing 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, Paisley, UK), 4 mM L-glutamine (Gibco, Paisley, UK), 2 mM sodium pyruvate (BDH, Poole, UK), and 10% heat-inactivated foetal calf serum (low endotoxin, Gibco, Paisley, UK). At this stage, 100 ng/ml of *E. coli* O55:B5 lipopolysaccharide (LPS; Sigma, Poole, UK) was added to one of the control tubes as a positive indicator of cytokine production. Cell suspensions were incubated at 37°C for 18 hr, centrifuged (1,000*g,* 15 min, room temperature) and the cell free supernatants removed and filter sterilised (0.2 μm: Sartorius, Göttingen, The Netherlands). They were then aliquoted and stored at −70°C until required for cytokine assay.

Infection of Ferrets and Removal of Blood Samples for Cytokine Assay

Adult male ferrets (approximately 1 kg) were anaesthetised under ether and infected intranasally with 10^6 EID_{50} of the appropriate virus as described previously [Toms et al., 1976]. Core body temperature was monitored using telemetric transmitters (Dataquest 4; Data-Sciences Inc., St. Pauls, Minnesota) implanted into the peritoneal cavity [von Itzstein et al., 1993]. At various times during pyrexia, blood samples were removed by cardiac puncture under isoflurane anaesthesia. Samples were transferred to heparinised tubes, centrifuged (1,000*g,* 10 min), and plasma stored at −70°C for cytokine assay.

Assay of Cytokines

For analysing the supernatants from the PBLs, commercial ELISA kits for human cytokines were used: IL-1β, IL-6, TNF-α, and IFN-γ were obtained from Genzyme (West Malling, UK); IL-8 and MIP-1α from R&D Systems (Abingdon, UK). Assays were carried out according to the manufacturer's instructions, diluting the samples if necessary with RPMI 1640. For measuring IL-1 and IL-6 in ferret plasma, biological assays were carried out by Dr. R. Scott (University of Newcastle Medical School, UK) as previously described [Hayes et al., 1994; Houde & Arora, 1990].

Examination of Recombinant Human Cytokines for Pyrogenicity in Rabbits

Recombinant human IL-1β, IL-6, and TNF-α (Genzyme, West Malling, UK) were diluted in complete RPMI (to give 100,000, 1,000, and 10 pg/ml) and stored at −70°C. The cytokine solutions were injected at 1 ml/kg into the marginal ear veins of adult male New Zealand white rabbits (3–4.5 kg). Core body temperatures were monitored using telemetric transmitters implanted in their peritoneal cavities. Pre-injection rabbit temperatures varied ± 0.22°C (S.D. 0.16), so only temperature rises above 0.4°C could be regarded as significant. Furthermore, temperatures were recorded for only 120 min, during which any temperature rises were likely to be caused by the injected cytokine and not by a secondary cytokine induced by the injected inoculum.

Prevention of Endotoxin Contamination

Previously adopted methods were used [Sweet et al., 1979]. Tissue culture media were freshly made, and disposable plastic pipettes and tubes used whenever possible. Glassware was heated to 160°C for a minimum of 2 hours, and solutions autoclaved for 2 hours.

Statistical Analysis of Data

Data were collated and analysed for statistical significance using the student's *t*-test.

TABLE I. Cytokine Levels Obtained From Stimulating PBLs With Either Clone 7a or A/Fiji*

	Stimulus	TNF-α	IL-1β	IL-6	IL-8	MIP-1α	IFN-γ
	Control	129 (22)	277 (26)	4112 (217)	47132 (3627)	386 (40)	0 (0)
Expt. 1	LPS	>1961 (55)	>1333 (0)	24599 (2338)	213469 (3666)	>3565 (0)	0 (0)
	Clone 7a	169 (1)[f]	276 (43)[d]	3152 (104)[a]	18272 (1210)[b]	1392 (61)[cd]	0 (0)
	A/Fiji	232 (0)[b]	468 (30)[b]	4825 (57)[a]	33189 (2677)[a]	1181 (31)[c]	0 (0)
	Control	188 (5)	114 (13)	5465 (321)	21012 (952)	194 (66)	0 (0)
Expt. 2	LPS	>2000 (0)	>1333 (0)	>39998 (0)	144365 (2014)	>3565 (0)	0 (0)
	Clone 7a	264 (11)[bf]	174 (13)[af]	4092 (228)[ae]	9139 (347)[b]	751 (16)[bf]	0 (0)
	A/Fiji	474 (3)[c]	688 (36)[c]	7425 (302)[b]	12973 (1467)[b]	1213 (9)[c]	0 (0)

*The levels are quoted as pg/ml of cytokines in supernatants from suspensions of PBL (2×10^6 cells/ml) stimulated with 100 EID_{50}/cell of either virus for 18 hr. Positive controls received 100 ng/ml of *E. coli* O55 lipopolysaccharide (LPS). Figures in parentheses indicate standard deviation. Figures for LPS stimulation are all significantly different from controls ($P \leq 0.01$).
Figures for clone 7a and A/Fiji.
[a]Differs from control $P \leq 0.05$.
[b]Differs from control $P \leq 0.01$.
[c]Differs from control $P \leq 0.001$.
[d]Differs from A/Fiji $P \leq 0.05$.
[e]Differs from A/Fiji $P \leq 0.01$.
[f]Differs from A/Fiji $P \leq 0.001$; only figures underlined [e.g., 169 (1)[f]] are not significantly different from controls.

RESULTS

Cytokine Levels in Supernatants of Virus Treated PBLs

Stimulation experiments were carried out several times, and supernatants from at least eight experiments were examined for cytokine content. Two aliquots of some supernatants were assayed independently and the results from both assays proved similar. Table I shows the results of assays of six cytokines in supernatants from two typical experiments. Such supernatants had induced previously different levels of fever (clone 7a high, A/Fiji low) when injected intracardially into ferrets [Jakeman et al., 1991]. All the cytokines, except IFN-γ, were liberated from PBLs on incubation alone, but, again with the exception of IFN-γ, the positive control (LPS) showed that far larger amounts of cytokines could be induced in the cells on stimulation. However, the effect of incubation with the two influenza viruses was relatively small and for all cytokines there was no evidence of clone 7a inducing substantially more cytokine than A/Fiji; indeed in some cases it was the reverse (Table I). Large amounts of IL-8 were released from PBLs on incubation alone, as has been noted by others [Schnyder-Candrian et al., 1995]. Although LPS caused an even greater release of IL-8, the two viruses had inhibitory effects. This inhibitory effect has been reported previously [Sprenger et al., 1996]; the reason for this is unknown.

Levels of IL-1 and IL-6 in Plasma Obtained From Infected Ferrets During the Course of Fever

Following intranasal infection of ferrets, the magnitude and duration of fever was greater with clone 7a than A/Fiji (Fig. 1a). The variable but low levels of IL-1 found in plasma samples of ferrets at the time of inoculation of virus did not increase during the febrile stage for either clone 7a or A/Fiji (Fig. 1b). In animals infected with A/Fiji, but not clone 7a, IL-1 levels rose significantly during defervescence and were highest once the fever had ceased. IL-6 levels were negligible in the plasma of ferrets prior to infection (Fig. 1c). In A/Fiji infected animals there was a small, but significant, rise in IL-6 levels during the fever, but this did not occur for clone 7a infected animals.

Pyrogenicity in Rabbits of Recombinant IL-1β, IL-6, and TNF-α

In these assays, rabbits were used to measure the fever produced by more than one cytokine. However, a suitable interval (at least 7 days) was allowed between each assay, and no animal received the same dose of cytokine twice. The responses to 10, 1,000, and 100,000 pg/kg of IL-1β, IL-6, and TNF-α are shown in Figure 2a–c respectively. All three doses of IL-1β produced fevers 1–2 hr postinjection which were higher than 0.4°C above the baseline (the normal variation of untreated animals). However, only the 100,000 pg/kg dose invoked a significant fever (Fig. 2a). IL-6 was nonpyrogenic; it is not clear why the 10 pg/kg dose showed a slight rise 90–120 min after injection (Fig. 2b). Only the high dose of TNF-α was pyrogenic (Fig. 2c).

DISCUSSION

Previously we failed to demonstrate a correlation between the levels of IL-1, IL-6, and TNF in supernatants derived from the interaction of PBLs with clone 7a or A/Fiji in vitro and the corresponding levels of pyrogenicity induced by the same supernatant in vivo [Jakeman et al., 1991]. In the current experiments, the levels of these three cytokines were similar in supernatants derived from interaction of clone 7a or A/Fiji with PBLs, and were similar to those described previously [Jakeman et al., 1991], although cytokine levels in control supernatants were higher. In addition, levels of two other pyrogenic cytokines (MIP-1α and IFN-γ) were similar for both viruses, and neither of them, nor LPS, could induce IFN-γ in the PBLs. Production of IL-8 was actually suppressed in comparison with levels liberated from unstimulated cells, which is in agreement with the results of others [Sprenger et al., 1996]. Overall, this recent comprehensive survey of cytokine

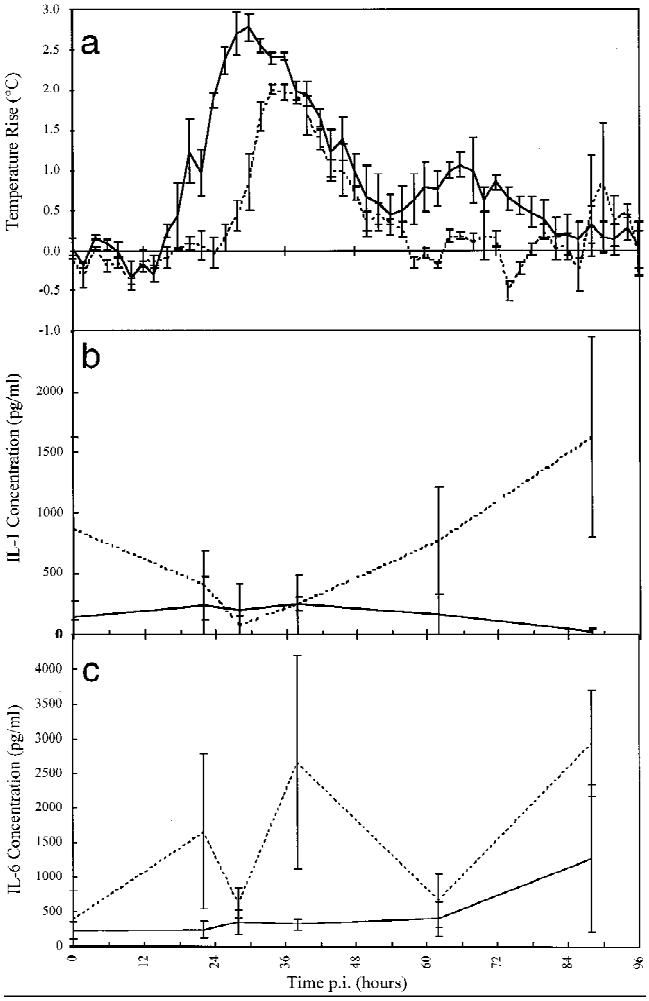

Fig. 1. Body temperature (**a**), IL-1 plasma concentration (**b**), and IL-6 plasma concentration (**c**) following intranasal infection of ferrets with 10^6 EID_{50} of clone 7a (———) or A/Fiji (–) influenza viruses. Error bars show standard deviation of the mean three animals.

production by the virulent (clone 7a) and attenuated (A/Fiji) viruses supports the conclusions of previous work, that no correlation could be established between the induced levels of known pyrogenic cytokines and production of fever in vivo.

These conclusions are also supported, at least for IL-1 and IL-6, by the fact that circulating levels of these two cytokines in infected ferrets could in no way be correlated with the onset or magnitude of the fevers induced by the two viruses (Fig. 1). However the bioasssays used for measuring IL-1 and IL-6 were designed for measurement of human and murine cytokines; it is possible that they are less sensitive for ferret cytokines.

The fever responses of rabbits to IL-1β described here (0.6–0.8°C rise, 60–120 min after injection) for 100,000 pg/kg (0.1 μg/kg) were larger and later than those detected in a previous study [Dinarello et al., 1986] (about 0.3°C rise around 50 min after injection). The response to TNF-α described here (about 0.6°C, 60–100 min after injection) for 100,000 pg/kg was similar in level, but later than that detected by Dinarello et al. (about 0.6°C rise around 45 min after injection). However there is reasonable agreement between the two studies considering we used intraperitoneal telemetry for detecting temperature rise, whereas Dinarello et al. [1986] used rectal temperatures, and the sources of recombinant cytokines were different in the two studies. In drawing conclusions from the experiments reported here and previously, direct comparisons cannot be made because different hosts and different methods were used for measuring fever. Nevertheless, it should be noted that in rabbits 100,000 pg/kg of IL-1β and TNF-α were needed to induce a detectable temperature rise, and that amount of TNF-α was not significantly pyrogenic; whereas the cytokines induced in supernatants of PBLs (2×10^6 cells/ml) by clone 7a and A/Fiji were often at levels much less than 5,000 pg/ml (Table I) [Jakeman et al., 1991]. In previous work, these supernatants produced appreciable fevers when 2 ml/kg were injected intracardially into ferrets [Jakeman et al., 1991]. Furthermore, less than 5,000 pg/ml of IL-1 and IL-6 were found in the plasma of ferrets

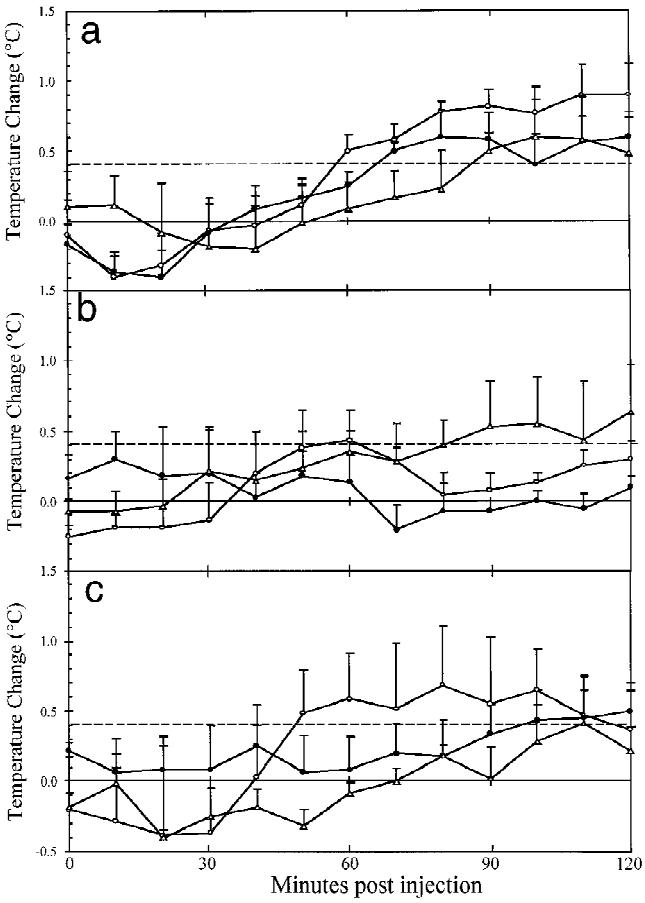

Fig. 2. Pyrogenicity of recombinant human cytokines in rabbits. Mean temperature response following injection of 100,000 (–○–) 1,000 (–●–) or 10 (–△–) pg/kg of IL-1β (**a**), IL-6 (**b**), or TNF-α (**c**) into three rabbits. Responses are plotted as deviation from mean baseline temperature. Error bars show S.E.M. Responses greater than 0.4°C above baseline (as shown by the dashed line) are significant (see Methods).

suffering high fevers during infection with the two viruses.

Overall, the conclusion of the work reported here and previously is that we can find no evidence that induction of known pyrogenic cytokines from monocytes/macrophages is responsible for the febrile response in influenza. The possibility that some other pyrogenic mediator(s) is produced by these or other host cells cannot be ruled out.

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